Inhibition of NFAT Signaling Restores Microvascular Endothelial Function in Diabetic Mice

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Adult heterozygous diabetic Akita (*Ins2*$^{+/-}$) mice were used (stock number 007562; D2.B6 background; The Jackson Laboratory, Maine). FVBN 9x-Nuclear Factor of Activated T-cells (NFAT)-luciferase reporter (NFAT-luc) mice[1-3] and Akita mice (stock number 003548, C57BL/6J background, The Jackson Laboratory) were used to generate Akita/NFAT-luc mice and non-diabetic wild-type (WT)/NFAT-luc littermates, backcrossed at least four generations into the C57BL/6J background. Animals had free access to tap water and were fed normal chow diet. Blood glucose (CONTOUR® meter; Bayer) and body weight were monitored weekly. Because of the limited hyperglycemia observed in Akita female mice, male mice were studied for all parts of the study except for the Kaplan-Meier survival curves in Figure 7E that includes data from both sexes. Mice were euthanized by cervical dislocation after anesthesia with 3% isoflurane in oxygen (2 L/min). For NFAT-luciferase experiments, mice were anaesthetized by intraperitoneal (i.p.) injection of 7.5 mg ketamine hydrochloride and 2.5 mg xylazine per 100 g body weight and euthanized by exsanguination through cardiac puncture. All animal protocols in this study were performed in accordance with UK Home Office regulations (Project License PIL60/4265) and the Malmö/Lund Animal Care and Use Committee (Permits M78-10, M29-12, and M9-15) and abided by the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament.

Laser Doppler imaging, iontophoresis and localized heating

The study protocol is shown in **Figure 1A**. Akita mice were randomized based on body weight to receive daily i.p. injections of the NFAT blocker A-285222 (0.29 mg/kg body weight) or vehicle (saline) for 4 weeks. Microvascular function was assessed blinded and non-invasively, before ("Pre"; at 7-12 weeks of age), directly after ("Post-1")- or 4 weeks after ("Post-2") the end of the treatment, using a laser Doppler imager (LDI; moorLDI, Moor Instruments Ltd, Axminster, UK) as previously described [4, 5]. In brief, 2 days prior to the measurements, fur was removed from the flank area of the mouse by shaving and with depilatory cream (Veet® Reckitt-Benckiser, Søborg, Danmark). General anesthesia was induced (5% isoflurane in 2 L/min oxygen) and maintained during the procedures (1.5-2% isoflurane in 1.5 L/min oxygen), and body temperature was maintained at 37°C using a heat mat.

For assessing microvascular responses to acetylcholine (ACh), an iontophoresis chamber (ION6 probe, Moor Instruments Ltd, Axminster, UK) was attached to the flank and a reference electrode pad attached to the underside of the animal to complete the iontophoresis circuit. To standardize basal perfusion, blood vessels were pre-constricted with iontophoresis of 1% phenylephrine (PE) for 5 min (current=100 μA), followed by iontophoresis of 2% ACh for 10 min (current=100 μA). At a different site on the opposite flank, 2% sodium nitroprusside (SNP) was iontophoresed for 10 min (current=100 μA). To determine the contribution of endothelium-derived nitric oxide (NO) to ACh-mediated vasodilatation, microvascular responses to ACh were assessed 30 min after i.p. injection of the non-selective inhibitor of NO synthase, N(G)-nitro-L-arginine methyl ester (L-NAME, Sigma Chemicals; 20 mg/kg). [4] On a separate day and to determine the maximum microvascular dilator capacity, a hyperemic response was initiated by localized heating of the skin using a specially designed heating probe (SH02™ skin heating unit and SHP3 probe, Moor Instruments Ltd, Axminster, UK). Perfusion was measured continuously as the temperature of the heating probe was increased to 42°C (1°C/min) and maintained for >10 min, which was sufficient for maximum vasodilatation to plateau.

The LDI scans continuously during the iontophoresis and localized heating periods and provides a measure of microvascular perfusion (in arbitrary perfusion units). Color-coded images were generated for each perfusion scan and numerical outputs were produced (moorLDI software version 5.2) and analyzed using proprietary software (Moor Instruments, version 5.3). The vascular response to ACh, PE and heating is presented as the percentage change from basal perfusion measured during 5 scans prior to the application of PE or initiation of heating. Endothelium-dependent and –independent vasodilation in response to ACh or SNP, respectively, was calculated as the difference between maximum vasodilation in response to ACh or SNP and maximum vasoconstriction in response to PE.

The response to localized heating was expressed as the percentage change in perfusion from baseline measurements to maximum vasodilation.

NFAT-dependent transcriptional activity

Diabetic Akita/NFAT-luc and non-diabetic WT/NFAT-luc littermate mice were euthanized at 4, 8, 12 and 24 weeks of age (N=6 mice/group) and skin harvested for measurements of NFAT-dependent transcriptional activity. A separate group of Akita/NFAT-luc and WT/NFAT-luc mice received daily i.p. injections of the NFAT blocker A-285222 (0.29 mg/kg body weight) or vehicle (saline) for 4 weeks (starting at 8-11 weeks of age). Mice were euthanized either directly after the treatment or 9 weeks after completion of the treatment. At termination, the aortic arch, abdominal aorta, carotid arteries and skin were collected and dissected free from surrounding tissue in ice-cold Ca^{2+}-free physiological saline solution (PSS; containing in mmol/L: NaCl, 135; KCl, 5.9; MgCl2, 1.2; Hepes, 11.6; glucose, 2.0; pH 7.4). Luciferase activity was measured in tissue homogenates as previously described [2]. Optical density was measured using a Tecan INfinite M200 instrument (Tecan Nordic AB, Mölndal, Sweden) and data is expressed as relative luciferase units (RLU) per µg of protein, as assessed using EZQ protein quantification kit (Molecular Probes, Invitrogen, Paisley, UK) or DC Protein Assay (Bio-Rad Laboratories, Sundbyberg, Sweden).

Histology and confocal microscopy

Skin biopsies from the flank of the mice were fixed overnight in 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.4), and either dehydrated and paraffin embedded for sectioning (5 µm) or processed as whole mounts for immunostaining. Sections were used for histology (hematoxylin and eosin; H&E), for quantification of NFATc3 nuclear accumulation using confocal microscopy or for immunohistochemistry of endothelial nitric oxide synthase (eNOS). For NFATc3 staining, sections were subjected to heat induced epitope retrieval using citrate buffer (10 mmol/L; pH 6.0) and stained as previously described.[1] Briefly, sections were permeabilized with 0.2% Triton X-100 in PBS, blocked for 2h with 2% bovine serum albumin (BSA) in PBS and incubated with rabbit polyclonal anti-NFATc3 (sc-8321, 0.2 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with Cy5 anti-rabbit IgG (3 µg/mL, Jackson Immuno Research, West Grove, PA) for 1h at room temperature. Nuclei were stained with SYTOX green (Molecular Probes, Invitrogen, Paisley, UK). For eNOS staining, sections were incubated with 0.3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase activity, then rinsed with PBS, blocked with 10% normal goat serum for 1h and incubated with rabbit polyclonal anti-eNOS antibody (0.125 µg/mL, sc-654 Santa Cruz Biotechnology) or non-immune rabbit IgG (0.125 µg/mL, ab27478, Abcam, Cambridge, UK) overnight at 4°C. Biotinylated goat anti-rabbit IgG antibody (0.75 µg/mL, BA-1000, Vector Laboratories, Burlingame, CA) and Vectastain Elite ABC kit (PK-6100, Vector laboratories, Burlingame, CA) were used for visualization. Sections were counterstained with Mayer's hematoxylin, examined at 100X (Nikon Eclipse E800 microscope; Nikon, Tokyo, Japan) and imaged with a Nikon DS-5Mc camera, using Nikon DS-U1 control unit and NIS-Elements v3.22.

Whole mounts were permeabilized with 0.5% Triton X-100 in PBS and blocked for 2h with 2% BSA in 0.2% Triton X-100 in PBS prior incubation with anti-NFATc3 (sc-8321, 0.2 µg/mL, Santa Cruz Biotechnology) or anti- cluster of differentiation 31 (CD31; sc-1506, 0.8 µg/mL, Santa Cruz Biotechnology) for 48 hours at room temperature, followed by incubation Cy5 anti-rabbit IgG (3 µg/mL; Jackson Immuno Research). Nuclei were stained with SYTOX green. Whole mounts and sections were examined at 20X and 63X, respectively using a Zeiss LSM 5 laser scanning confocal microscope. Specificity of immune staining was confirmed by the absence of staining when primary antibodies were omitted from the protocol. NFATc3 nuclear accumulation in skin microvessels was quantified as previously described. [2, 6, 7] Briefly, for scoring of NFATc3-positive nuclei in vessels, multiple fields for each skin section were analyzed under blinded conditions. A cell was considered positive if co-localization was observed in the nucleus, whereas a cell was considered negative if no

co-localization was visualized. A minimum of 100 cells per animal were inspected. For visualization of co-localized image regions or double tagged regions (red: NFATc3 tagged with Cy5 and green: nuclear regions tagged with SYTOX Green), the crosshair function of the LSM program was used. This tool leads to the distribution of all image pixels over 4 quadrants in a scattergram according to their intensity levels, with the background pixels sorted into the bottom left quadrant, the single-tagged pixels (either red or green) into the upper left and bottom right quadrants, and the pixels having an intensity above the background in both channels (i.e., co-localized pixels) represented by the upper right quadrant. The image pixels corresponding to the upper right quadrant are then color-coded white in the original image to allow fast identification of co-localized areas.

Skin organ culture and enzymatic dissociation of dermis and epidermis

Two days after removing the fur by shaving and with depilatory cream (Veet® Reckitt-Benckiser), skin from the flank of WT/NFAT-luc mice was dissected out, cleaned from fat and muscle and cut into pieces (~2-3 x 2-3 mm). These were cultured with or without A-285222 (1 µmol/L) in DMEM (Dulbecco's modified minimal essential medium; F0405; Biochrom GmbH, Berlin, Germany) supplemented with 5 mmol/L glucose, 200 µg/mL BSA, 50 U/mL penicillin and 50 µg/mL streptomycin, at 37°C for various time-points up to 48 hours as indicated in the figure legends, according to a protocol that has been shown to preserve human and rodent skin structure and function for extended periods of time. [8, 9] After organ culture, the skin was frozen in liquid nitrogen and stored at -80°C until enzymatic dissociation of dermis and epidermis according to a previously described protocol. [10, 11] Briefly, whole skin was incubated with dispase II (5 µg/mL; Gibco Life Technology, Stockholm, Sweden) for 45 minutes at 37°C, after which fractions were manually dissected under a light microscope. In order to test for potential effects of the enzymatic dissociation on protein quantities or integrity, the method was compared to manual microdissection without prior enzymatic incubation and no differences were observed (data not shown). For western blot experiments to assess eNOS and p-eNOS (see below), microdissection only was used given that the method proved to be more time efficient when processing large number of samples.

RNA isolation, quantitative RT-PCR and absolute copy number of NFAT isoforms

Total RNA was isolated from skin (whole skin and dissociated dermis and epidermis as explained above) and thymus using TRI Reagent (Sigma Aldrich, Stockholm, Sweden) as previously described [12]. cDNA was synthesized with RevertAid First Strand cDNA kit, (Thermo Fisher Scientific, Stockholm, Sweden) and amplified using TaqMan gene expression assays for mouse eNOS Mm00435217_m1, a custom designed assay for luciferase mRNA (forward primer AACTGCCTGCGTCAGATTCTC, reverse primer AGTATCCGGAATGATTTGATTGC, FAM-labelled probe ATGCCAGGGATCCTA), OPN (Mm00436737_mL) and IL-6 (Mm00446190_ml) on a Viia7 instrument (Applied Biosystems, Carlsbad, CA). Target gene expression was normalized to HPRT Mm00446968_m1 and cyclophilin B Mm00478295_m1 and relative mRNA quantity was calculated using the comparative threshold method ($\Delta\Delta C_t$). All reactions were performed in triplicates.

For the absolute quantification of NFAT isoform expression, thymus cDNA was amplified using TaqMan gene expression assays for NFATc1 Mm00479445_m1; NFATc2 Mm01240679_m1; NFATc3 Mm01249200_m1; NFATc4 Mm00452375_m1 and β-actin Mm00607939_s1. PCR fragments were isolated using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and concentrations were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE). The number of copies per µl for each NFAT isoform and for β-actin was calculated using the formula [X * $6.022x10^{23}$ molecules/mole] / [N * 660 g/mole] where X is the concentration of isolated PCR fragments in g/µl and N is the length of the PCR product in base pairs. [13] Ten-fold serial dilutions from 10^9 to 10 copies per µl were prepared and amplified in triplicates to generate absolute standard curves for each isoform, from where copy numbers for dermis

and epidermis samples, expressed per µg of total RNA input, were determined by reading off their threshold cycle (Ct) values after amplification in triplicates.

Western Blot

Whole skin, dermis and epidermis were homogenized using a glass tissue grinder (Wheaton, Fischer Scientific, Sweden) and proteins were extracted using TRI Reagent (Sigma Aldrich, Stockholm, Sweden) according to the manufacturer's instructions. Protein concentration was determined using EZQ protein quantification kit (Molecular Probes, Invitrogen, Paisley, UK) and an equal amount of protein was loaded in 4-15% Mini Protean TGX Stain-free gels (Bio-Rad Laboratories, Sundbyberg, Sweden) and transferred to 0.2 µm PVDF membranes using Trans-Blot turbo (Bio-Rad Laboratories, Sundbyberg, Sweden). Membranes were probed overnight at 4°C with primary antibodies against CD31 (sc-1506, 1 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) and α-tubulin (A01410, 0.1 µg/mL, GenScript Corporation, Piscataway, NJ) with horseradish peroxidase-conjugated secondary antibodies (1:10,000; Cell Signal Technology, Danvers, MA). Labeling was detected by chemiluminescence (Supersignal West Dura, Thermo Scientific, MA).

For quantification of eNOS and p-eNOS, dermis was processed using a OMNI TH tissue homogenizer (OMNI International, Kennesaw GA, US) and proteins were extracted using TRI Reagent as described above. Proteins were separated on 4-15% TGX Stain-free gels and transferred to 0.45 µm low-fluorescence PVDF membranes. Membranes were then imaged for total protein quantification and probed overnight at 4°C with primary antibodies against eNOS (sc-376751, 0.4 µg/mL Santa Cruz Biotechnology, Santa Cruz, CA) or p-eNOS (Ser1177, sc-81510, 0.1 µg/mL Santa Cruz Biotechnology, Santa Cruz, CA). Data quantification was performed using Image Lab Software (Life Science Research, Bio-Rad Laboratories, Sundbyberg, Sweden). Signal intensity was normalized to total protein measured in the membrane after transfer. For each animal, dermis from 3-4 pieces of skin, each of~2-3 x 2-3 mm, were pooled to generate a data point.

Plasma cytokines, endothelial activation markers and total NO

Plasma levels of cytokines [interleukin (IL)-1α, IL-6, IL-10 and osteopontin (OPN)] and endothelial activation markers [soluble intercellular adhesion molecule 1 (sICAM), E-selectin, endothelin-1 (Et-1)] were measured in blood samples taken from the tail vein before and after A-285222 treatment (Pre, Post-1 and Post-2) Plasma cytokine levels were measured using the Bio-Plex™ multiplex ELISA system (Bio-Rad Laboratories, Hertfordshire, UK) and sICAM, E-selectin, Et-1and OPN were measured using Quantikine Colorimetric sandwich ELISA assays (R&D Systems, Bio-Techne Ltd, Abingdon, UK). Total NO related species (nitrite, nitrate, nitrosothiols) were measured 4 weeks after treatment with A-285222 (Post-2) using a gas phase chemiluminescence reaction of NO with ozone using Sievers nitric oxide analyzer (NOA) model 280i (Analytix, Bodon, UK).

Blood pressure

Blood pressure was assessed in conscious mice four weeks after treatment with A-285222 (Post-2) using a computerized tail-cuff non-invasive blood pressure system (CODA; Kent Scientific, Connecticut, USA). Before measurements were initiated, mice were adapted to the apparatus for at least 5 days.

Urinary albumin measurements

Urine was collected from the bladder at termination. Urinary albumin excretion was measured using an indirect competitive ELISA (Albuwell M, Exocell, Philadelphia, PA) and urinary creatinine concentration was measured using a picric acid assay (Creatinine Companion; Exocell); all according to the manufacturers' instructions.

Chemicals

A-285222 was kindly provided by Abbott Laboratories (Abbott Park, IL). All other chemicals were from Sigma Aldrich unless otherwise specified.

Statistics

Results are expressed as means ± SEM unless otherwise specified. Statistical significance was determined using Student's t-test and one or two-way ANOVA followed by Dunnett's multiple comparisons or Bonferroni *post hoc* tests. For survival analysis Kaplan-Meier curves were generated from data compiled throughout the study and Mantel-Cox log-rank test performed to compare survival between groups. Statistical analyses were performed using SPSS version 14.1 or GraphPad software (Prism 6.0 and 7.0).

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